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# Porphyrin−Apidaecin Conjugate as a New Broad Spectrum Antibacterial Agent

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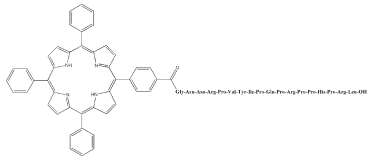
**ABSTRACT** The conjugation of the cationic antimicrobial peptide, apidaecin Ib, to the anionic photosensitizer, 5(4′-carboxyphenyl)-10,15,20-triphenylporphyrin (cTPP), afforded a new antibacterial agent effective, under light activation, against both Gram-positive and Gram-negative bacteria. At low concentrations (1.5−15 $\mu$M) the conjugate was able to reduce the survival of *Escherichia coli* cells by 3−4 $\log_{10}$, and most notably, it resulted photoactive also against hard-to-treat *Pseudomonas aeruginosa*, although at higher concentration (60 $\mu$M). Under similar conditions, the photosensitizer alone was only photoactive against *Staphylococcus aureus* while the unconjugated peptide was inactive against all the bacterial strains tested. This study shows the possibility of obtaining new broad-spectrum apidaecin−photosensitizer conjugates with potent antibacterial activity.

**KEYWORDS** Antimicrobial PDT, antimicrobial peptides, porphyrin, apidaecin, photosensitizer−peptide conjugate



The worldwide rise of antibiotic resistance stimulates the search for new strategies for controlling bacterial infections based on the use of agents different from antibiotics.[1] Photodynamic therapy (PDT) is a promising approach to the treatment of superficial and localized infections and can be particularly useful in the dermatological field. PDT uses a visible light absorbing molecule, called photosensitizer, that can be activated by irradiation with visible light and, in the presence of oxygen, generates cytotoxic reactive oxygen species (ROS). It is well established that singlet oxygen ($^1O_2$) is produced as the main species responsible for cell death in PDT.[2−4] It has been demonstrated that Gram-positive bacteria can be efficiently killed by light after their incubation with a number of photosensitizers. On the contrary Gram-negative bacteria are less susceptible to photodynamic killing, and only cationic photosensitizers can bind efficiently to this type of bacteria and induce their photoinactivation.[5] During PDT multiple cellular targets are damaged, and this strongly reduces the probability of developing the resistance phenomena which frequently occur after repeated antibiotic treatments.[6]

Similarly, there is evidence that small cationic antimicrobial peptides (CAMPs), which are components of the innate defense of many organisms, are not prone to elicit resistance mechanisms as almost all classes of antibiotics do.[7] CAMPs are naturally occurring peptides involved in the first immune response against infections, and are present in nearly all living organisms. Among CAMPs, the family of small proline- and arginine-rich peptide is being extensively studied because of some unique features, such as good antimicrobial activity and capability of entering target cells by a non-pore-forming mechanism (not yet fully elucidated).[8] Apidaecin 1b belongs to this class of CAMPs and exerts a good antimicrobial activity against several Gram-negative bacteria, at concentrations that are not toxic to eukaryotic cells.[9,10] Recently, we have shown that apidaecin can translocate a neutral fluorescent cargo into a bacterial cell, preserving most of its antibacterial activity.[10] As a consequence we wonder if apidaecin can behave as a molecular vector to transport a photosensitizer into a bacterial cell, where, by light activation, it can generate ROS and induce cell death by photooxidative damage of various cellular components.

In this communication we report the synthesis, characterization and antibacterial activity of a new photosensitizing agent obtained by conjugating the cTPP to apidaecin 1b. Our aim was to investigate the possibility of obtaining a photosensitizing agent with a broader spectrum of activity and reduced drawbacks in comparison with the single components.

The synthesis of apidaecin 1b and the apidaecin−porphyrin conjugate (T-api) was performed by standard solid phase peptide synthesis. cTPP was covalently linked to the N-terminus of apidaecin. Contrary to cTPP, which is rather hydrophobic and can be dissolved in aqueous solvent only from a DMSO stock, the cTPP−apidaecin conjugate could be easily solubilized in aqueous solvent. The UV−visible absorption spectrum of cTPP conjugated to apidaecin did

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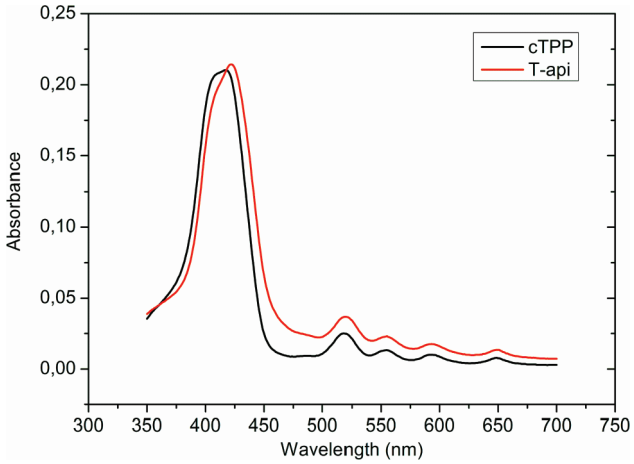


**Figure 1.** UV−visible absorption of 1.5 $\mu$M cTPP and T-api in phosphate buffered saline at pH 7.2.

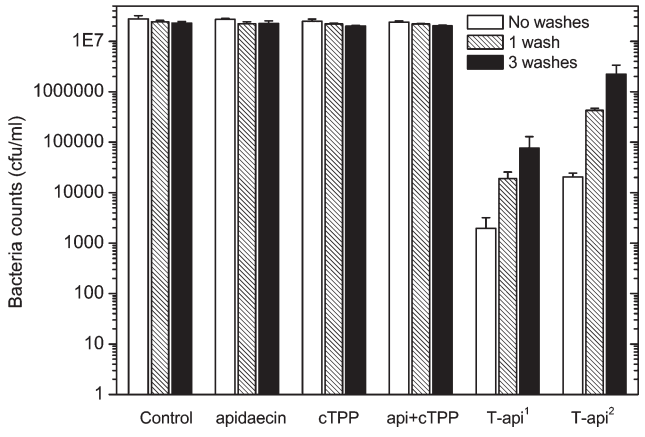


**Figure 2.** Survival of *E. coli* irradiated with 13.4 J/cm$^2$ of blue light after incubation with 15 $\mu$M apidaecin or cTPP, alone or in combination (api + cTPP), and T-api 1.5 $\mu$M (T-api$^2$) or 15 $\mu$M (T-api$^1$).

not change significantly in comparison to the unconjugated cTPP (Figure 1).

The antibacterial activity of T-api was first assessed without activation with light, by determining the MIC (minimal inhibitory concentration).[11] The results were compared to those of the unconjugated apidaecin and of the photosensitizer alone. Three bacterial strains were tested: the Gram-negative *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 25668, and the Gram-positive methicillin-resistant *Staphylococcus aureus* ATCC BAA 44. In agreement with previous studies,[12] apidaecin was active against *E. coli* with a MIC value of 8−16 $\mu$M, but resulted inactive against *P. aeruginosa* and *S. aureus*, at least up to 128 $\mu$M concentration. cTPP and the apidaecin conjugate proved to be inactive against all the strains tested, showing MIC values higher than 128 $\mu$M. The loss of antibacterial activity of the conjugate compared to apidaecin suggests that the size of the cargo (cTPP), appended to the peptide N-terminus, prevented the uptake of T-api into the bacterial cells, where the peptide is supposed to meet its ultimate target.[9] This observation does not exclude that the conjugated cTPP, activated by light, is able to kill bacteria, providing that T-api binds efficiently to the cells. Therefore, the phototoxicity of T-api was evaluated by determining the survival of bacterial cells exposed to 13.4 J/cm$^2$ of blue light after incubation for 60 min in the dark with the conjugate. Colony counting showed that irradiation of *E. coli* cells, treated with increasing concentrations of the conjugate (1.5 and 15 $\mu$M), caused a 3−4 $\log_{10}$ reduction of the colony forming units (cfu) (Figure 2). Conversely neither cTPP nor the peptide, alone or together, showed bactericidal activity upon illumination at the tested concentration (15 $\mu$M). The lack of bactericidal activity of apidaecin at the MIC concentration, upon illumination and in the dark (data not shown), can be explained considering that our photosensitization studies were performed in phosphate buffered saline (PBS) at high ionic strength (150 mM NaCl), which is known to depress the antibacterial activity of cationic peptides and of apidaecin in particular.[13,14] It is worth mentioning that, in our studies, bacteria in the stationary phase of growth, which are much more resistant to the activity of CAMPs than log-phase bacteria, have been used. Additional factors such as the *E. coli* strain, the cell density of the suspension, and the time of incubation with the bacteria may explain the difference between our results and those reported by others on bactericidal activity of apidaecin.[15]

The phototoxic activity of T-api was only partially reduced by repeated washings of *E. coli* cells before irradiation (Figure 2), suggesting that the conjugate was efficiently retained by the cells. The fluorescence microscopy analyses further supported this observation showing a strong red fluorescence signal of the cells incubated with T-api but not with cTPP (Figure 3).

The photoactivity of T-api was tested also against *P. aeruginosa*, which is rather insensitive to many antibacterial treatments. As expected the concentration of T-api necessary for inducing significant photokilling of *P. aeruginosa* was higher than that of *E. coli* (Figure 4). Two $\log_{10}$ reduction of cfu/mL was obtained with 60 $\mu$M T-api while no photokilling could be measured for cells irradiated after incubation with unconjugated cTPP and/or apidaecin. At this concentration, T-api bound to *P. aeruginosa* cells quite strongly because the photokilling efficiency was only slightly reduced by washing the cells one or three times before illumination and the red fluorescence in cells incubated with T-api was more intense than that of cells incubated with cTPP (Figure 3).

These results show that the conjugation of a porphyrin to the antimicrobial peptide apidaecin, promoting the interaction of the photosensitizer to Gram-negative bacteria, extends the efficacy of PDT also to this type of bacteria. Further studies will be necessary to localize the bacterial site where the photokilling activity is produced.

Gram-positive bacteria can be efficiently inactivated by neutral and anionic photosensitizers, like cTPP. As expected, *S. aureus* cells incubated with cTPP (1.5 $\mu$M) showed an about 4 $\log_{10}$ reduction of cfu/mL, after illumination with 13.4 J/cm$^2$ of blue light (Figure 5).

Under the same conditions, T-api proved to be more active than cTPP against *S. aureus*, inducing a further reduction of the bacterial survival, both in unwashed and washed cells. As fluorescence microscopy studies showed a comparable



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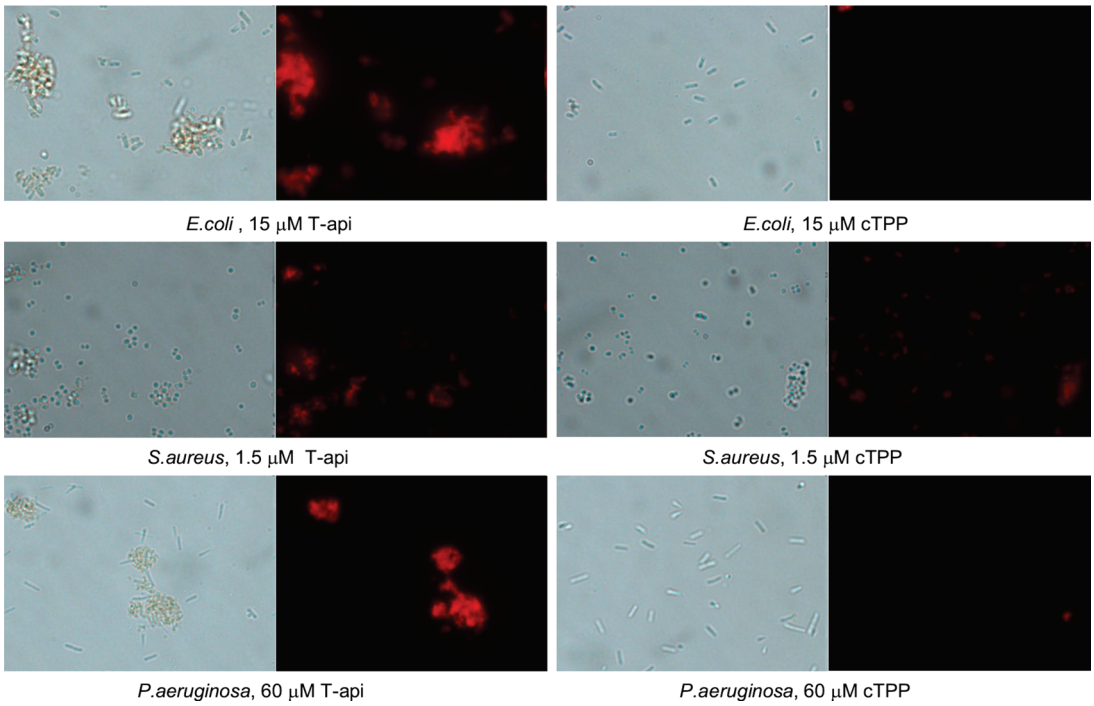


**Figure 3.** Fluorescence and corresponding bright field images of *E. coli*, *S. aureus* and *P. aeruginosa* incubated for 60 min with the indicated concentrations of T-api (left panels) or cTPP (right panels).

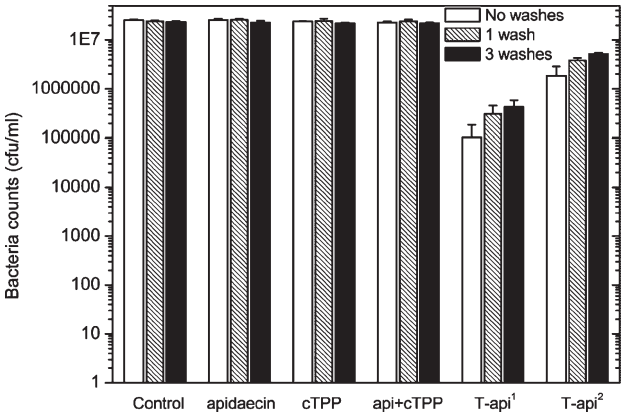


**Figure 4.** Survival of *P. aeruginosa* irradiated with 13.4 J/cm$^2$ of blue light after incubation with 60 $\mu$M apidaecin or cTPP alone or in combination (api + cTPP) and T-api 15 $\mu$M (T-api$^2$) or 60 $\mu$M (T-api$^1$).

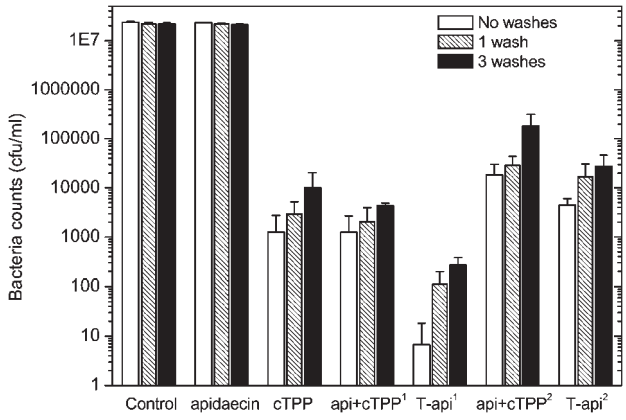


**Figure 5.** Survival of *S. aureus* irradiated with 13.4 J/cm$^2$ of blue light after incubation with 1.5 $\mu$M apidaecin or cTPP alone or in combination (api + cTPP) and T-api 0.15 $\mu$M (T-api$^2$) or 1.5 $\mu$M (T-api$^1$).

fluorescence of *S. aureus* after incubation with cTPP or T-api (Figure 3), the increased photosensitivity of *S. aureus* toward the conjugate could be ascribed to the ability of T-api to reach a site critical for bacterial survival, other than to higher uptake of the conjugate into the cell. Additional investigations will clarify this point.

In conclusion our results demonstrated the validity of our approach for obtaining antibacterial agents with a broad spectrum of activity. As far as we known, this is the first time that a cationic peptide active against some Gram-negative bacteria is conjugated to a photosensitizing molecule, mainly photoactive against Gram-positive bacteria. The antibacterial activity of the conjugate was higher than that of the individual components and, most notably, with a broader spectrum of activity. Further studies are ongoing which are aimed at optimizing the antibacterial activity of photosensitizer−peptide conjugates by considering different sites of conjugation in the peptide and photosensitizers of different chemical structure.

## EXPERIMENTAL PROCEDURES

The synthesis of both apidaecin 1b and the apidaecin−porphyrin conjugate (T-api) was performed by standard Fmoc/HBTU chemistry.[16,17] cTPP, synthesized according to the literature,[18] was covalently linked to the N-terminus of apidaecin when the peptide was still attached to the resin. The peptides were side-chain deprotected and cleaved from the solid support with 95% trifluoroacetic acid, providing the crude



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products in about 80% purity. Apidaecin and its cTPP conjugate were obtained after purification by semipreparative HPLC with ≥95% homogeneity, and they were characterized by analytical HPLC and ESI-MS (see Supporting Information).

Bacteria in the stationary phase of growth were collected from overnight cultures and resuspended in phosphate buffered saline (PBS). Bacteria (~$2 \times 10^7$ cell/mL) were incubated for 60 min in the dark with cTPP, apidaecin or T-api and illuminated for 20 min to give 13.4 J/cm$^2$ of blue light, with or without the washings of the cells. Appropriate control samples were processed in parallel. The effect of the treatment was evaluated by plating treated and untreated cell samples onto brain heart infusion (BHI) agar and counting the cfu/mL after overnight incubation at 37 °C.

**SUPPORTING INFORMATION AVAILABLE** Detailed experimental procedures on peptides and conjugate synthesis and protocols of bacterial treatments. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Author Contributions:** M.G. and E.R. conceived and designed the project; S.C. performed the synthesis of cTPP; E.B. performed the synthesis and purification of apidaecin and its conjugate; R.D. performed the experiments on bacterial inactivation and fluorescence microscopy and participated in writing of the manuscript; E.R. participated in the experimental design, data analysis, coordination. M.G. and E.R. wrote the manuscript.

**Notes:** The authors declare no conflict of interest.

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**ABBREVIATIONS** CAMP, cationic antimicrobial peptide; cfu, colony forming unit; cTPP, 5(4′-carboxyphenyl)-10,15,20-triphenylporphyrin; DMSO, dimethyl sulfoxide; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, 2-[1*H*-benzotriazol-1-yl]-1,1,3,3-tetramethyluronium hexafluorophosphate; MIC, minimal inhibitory concentration; PBS, phosphate buffered saline; PDT, photodynamic therapy; ROS, reactive oxygen species; T-api, apidaecin−porphyrin conjugate.

## REFERENCES

(1) Fischbach, M. A.; Walsh, C. T. Antibiotics for emerging pathogens. *Science* **2009,** *325*, 1089–1093.
(2) Sharman, W. M.; Allen, C. M.; van Lier, J. E. Photodynamic therapeutics: basic principles and clinical applications. *Drug Discovery Today* **1999,** *4*, 507–517.
(3) Hamblin, M. R.; Hasan, T. Photodynamic therapy: a new antimicrobial approach to infectious disease? *Photochem. Photobiol. Sci.* **2004,** *3*, 436–450.
(4) Maisch, T. A new strategy to destroy antibiotic resistant microorganisms: antimicrobial photodynamic treatment. *Mini-Rev. Med. Chem.* **2009,** *9*, 974–983.
(5) Malik, Z.; Ladan, H.; Nitzan, Y. Photodynamic inactivation of Gram-negative bacteria: problems and possible solutions. *J. Photochem. Photobiol., B* **1992,** *14*, 262–266.
(6) Lauro, F. M.; Pretto, P.; Covolo, L.; Jori, G.; Bertoloni, G. Photoinactivation of bacterial strains involved in periodontal diseases sensitized by porphycene-polylysine conjugates. *Photochem. Photobiol. Sci.* **2002,** *1*, 468–470.
(7) Jenssen, H.; Hamill, P.; Hancock, R. E. W. Peptide antimicrobial agents. *Clin. Microbiol. Rev.* **2006,** *19*, 491–511.
(8) Li, W. F.; Ma, G. X.; Zhou, X. X. Apidaecin-type peptides: biodiversità, structure-function relationships and mode of action. *Peptides* **2006,** *27*, 2350–2359.
(9) Castle, M.; Nazarin, A.; Yi, S. S.; Tempst, P. Lethal effect of apidaecin on *Escherichia coli* involve sequential molecular interaction with diverse targets. *J. Biol. Chem.* **1999,** *274*, 32555–32564.
(10) Gobbo, M.; Benincasa, M.; Bertoloni, G.; Biondi, B.; Dosselli, R.; Papini, E.; Reddi, E.; Rocchi, R.; Tavano, R.; Gennaro, R. Substitution of the arginine/leucine residues in apidaecin Ib with peptoid residues: effect on antimicrobial activity, cellular uptake, and proteolytic degradation. *J. Med. Chem.* **2009,** *52*, 5197–5206.
(11) Clinical and Laboratory Standards Institute. *Methods for dilution antimicrobial susceptibility test for bacteria that grows aerobically*, 7th ed.; Approved standard M7-A7; Clinical and Laboratory Standards Institute: Wayne, PA, 2006.
(12) Gobbo, M.; Biondi, L.; Filira, F.; Gennaro, R.; Benincasa, M.; Scolaro, B.; Rocchi, R. Antimicrobial peptides: synthesis and antibacterial activity of linear and cyclic drosocin and apidaecin 1b analogues. *J. Med. Chem.* **2002,** *45*, 4494–4504.
(13) Hancock, R. E. W.; Sahl, H.-G. Antimicrobial and host defence peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **2006,** *24*, 1551–1557.
(14) Casteels, P.; Tempst, P. Apidaecin-type peptide antibiotics function trough a non-pore forming mechanism involving stereospecificity. *Biochem. Biophys. Res. Commun.* **1994,** *199*, 1551–1557.
(15) Mardones, G.; Venegas, A. Chromogenic plate assay distinguishing bacteriolytic from bacteriostatic activity of antibiotic agent. *J. Microbiol. Methods* **2000,** *40*, 199–206.
(16) Sheppard, R. The fluorenylmethoxycarbonyl group in solid phase synthesis. *J. Peptide Sci.* **2003,** *9*, 545–552.
(17) Chan, W. C.; White, P. D. Basic procedures. In *Fmoc solid phase peptide synthesis: a practical approach*; Chan, W. C., White, P. D., Ed.; Oxford University Press: New York, 2000; pp 41−74.
(18) Matile, S.; Berova, N.; Nakanishi, K.; Fleischhauer, J.; Woody, R. W. Structural studies of exciton coupled circular dichroism over a large distance: porphyrin derivatives of steroids, dimeric steroids and brevetoxin B. *J. Am. Chem. Soc.* **1996,** *118*, 5198–5206.



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